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REVIEW

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Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation

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Abstract The large daily rhythm in circulating melatonin levels is a highly conserved feature of vertebrate physiology: high values always occur at night. The dynamics of the rhythm are controlled by the next-to-last enzyme in melatonin synthesis (serotonin → *N*-acetylserotonin → melatonin), arylalkylamine *N*-acetyltransferase (AANAT), the "melatonin rhythm enzyme". In vertebrate biology, AANAT plays a unique time-keeping role as the molecular interface between the environment and the hormonal signal of time, melatonin. This chapter describes the mammalian AANAT regulatory system, which includes the retina, neural structures, transsynaptic processes, and molecular events. In addition, special attention is paid to the functional characteristics of the systems which insure that the nocturnal increase in melatonin is an accurate and reliable indicator of the duration of the night, and why the melatonin rhythm is the most reliable output signal of the Mind's Clock.

Keywords Pineal gland · Melatonin · Arylalkylamine *N*-acetyltransferase · Circadian · Second messenger · Signal transduction

Introduction

The daily rhythm in melatonin is a conserved feature of vertebrate physiology, with high values always occurring at night in the dark and never during the day. This signal plays an important role in physiology, especially in those species in which seasonal changes in reproduction are regulated by seasonal changes in environmental lighting (Karsh et al. 1991; Arendt 1995; Barrell et al. 2000). In addition, melatonin can play an entraining role in circadian physiology (Redman et al. 1983; Arendt 1995; Sack et al. 2000; Pévet et al. 2002).

The precision and reliability of the photoneuroendocrine transduction which regulates the melatonin production system, the melatonin rhythm generating system, is determined by mechanisms which operate at several levels to insure integrity of the melatonin signal. The molecular interface between regulation and melatonin synthesis is the next-to-last enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT; Fig. 1). This chapter describes the melatonin rhythm generating system in mammals, highlighting features that insure accuracy and reliability.

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Dynamics of pineal indoles

Daily rhythms

Large daily rhythms in indole metabolism are a defining characteristic of the pineal gland and a notable feature of vertebrate circadian biology (Fig. 1). Pineal serotonin (5-HT) levels are higher during the day than at night; in some mammals the day/night ratio is greater than 10:1. Conversely, pineal *N*-acetylserotonin (NAS) and melatonin levels are low during the day and high at night (Klein 1974; Illnerová et al. 1978; Illnerová 1991). The switch between the day and night profiles of pineal indoles are driven by changes in the activity of AANAT, which increases at night 10- to 100-fold (Klein and Weller 1970; Klein 1985; Klein et al. 1997). This change in activity is due to an increase in enzyme protein and to activation mechanisms. Increased AANAT protein and activity reduces the abundance of 5-HT and increases the abundance of the product NAS. Increased levels of NAS increase the formation of melatonin by a mass action effect mediated by hydroxyindole-*O*-methyltransferase. This enzyme exhibits relatively small or no day/night variation (Wurtman et al. 1963; Klein and Lines 1969; Wartman et al. 1969; Klein 1974; Sugden and Klein 1983; Namboodiri et al. 1985; Ribelayga et al. 2000).

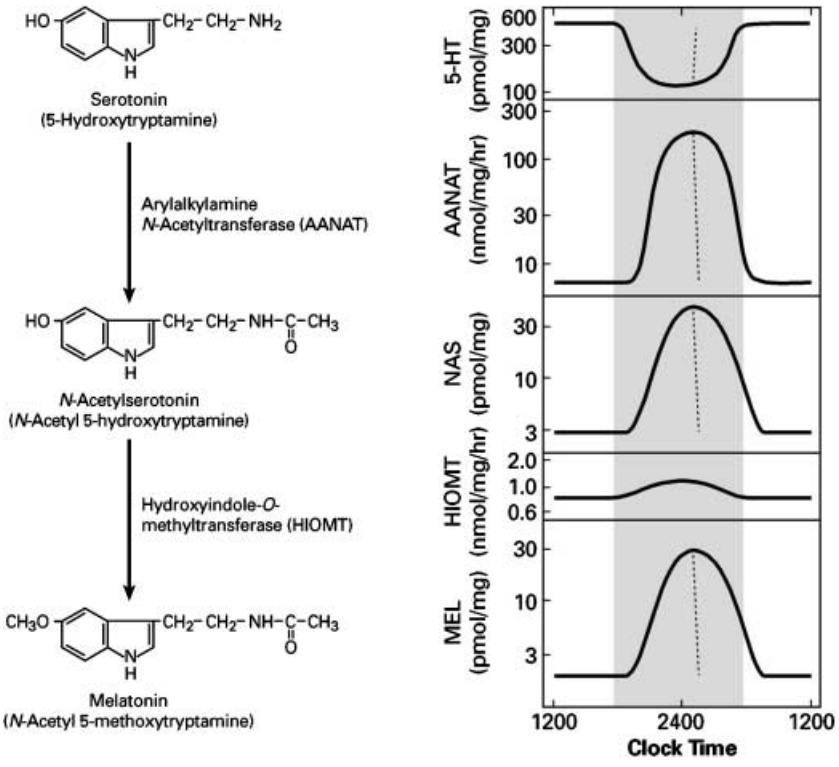

Fig. 1 Daily rhythm in pineal indoles. Serotonin (5-hydroxytryptamine; *5-HT*) levels are high during the day and decrease at night (*dark panel*) because of a large increase in arylalkylamine *N*-acetyltransferase (*AANAT*) protein and activity. AANAT transfers an acetyl group from acetyl CoA to 5-HT. The increase in AANAT activity results in an increase in the intracellular concentration of *N*-acetylserotonin (*N*-acetyl 5-hydroxytryptamine; *NAS*), which is converted to melatonin (*N*-acetyl 5-methoxytryptamine; *MEL*) by hydroxyindole-*O*-methyltransferase (*HIOMT*). Exposure to light at night causes a return to the day time pattern (*dotted line*). Modified after Klein (1974)

Accordingly, the cellular rate of *O*-methylation is largely a function of substrate availability, whereas the *N*-acetylation step is regulated by the amount of active AANAT protein. The major features of the 24-h pattern of pineal indole metabolism outlined in Fig. 1 are conserved among mammals and all other vertebrates.

Although it is clear that large changes in AANAT activity are responsible for the daily rhythm in pineal indoles, it is not clear what limits the minimum daytime and maximal nighttime rates of melatonin production. The determining factor could be the availability of cofactors, uptake of tryptophan, or the activity of other enzymes required for the conversion of tryptophan to melatonin (Klein and Weller 1970; Kapatos et al. 1981; Furukawa et al. 1993; Ribelayga et al. 2000; Martinez et al. 2001).

Rapid effects of light

A striking feature of pineal indole metabolism is the exquisite sensitivity it exhibits to light exposure at night, which causes remarkably rapid changes (Fig. 1). The critical perturbation is a rapid decrease in AANAT activity ($t_{1/2}$ ca 3 min; Klein and Weller 1972). This leads to a sharp decrease in the conversion of 5-HT to NAS and melatonin, resulting in a return to the daytime pattern of metabolites and level of circulating melatonin (Illnerová 1971; Illnerová et al. 1979; Mefford et al. 1983; Namboodiri et al. 1985).

The relationship between melatonin synthesis and circulating melatonin

Melatonin is highly lipophilic and is not stored at significant levels. Accordingly, it is released into the blood immediately upon synthesis. This close relationship between production and release is one of two factors that explain why rapid changes in melatonin synthesis are rapidly translated into similar changes in circulating levels of melatonin (Illnerová et al. 1978). The second is hepatic uptake, 6-hydroxylation, and subsequent metabolism (Kopin et al. 1961; Iguchi et al. 1982). Accordingly, circulating melatonin levels reflect a dynamic balance of production-regulated release and rapid hepatic destruction:

$$\xrightarrow{Production,release} Circulating\ melatonin \xrightarrow{Hepatic\ destruction}$$

The neural circuit regulating rhythms in pineal metabolism

All circadian systems include a circadian oscillator, a photodetector, and an output signal. In the case of the melatonin rhythm generating system, these elements and the neural connections linking them have been described in detail (for review, see Klein et al. 1991). This complete description makes the melatonin rhythm generating system unique among mammalian circadian systems.

The oscillator in the melatonin rhythm generating system is located in the suprachiasmatic nuclei (SCN; see Gillette and Mitchell 2002), the master circadian os-

cillator, also described as the Mind's Clock (Klein et al. 1991). The SCN control the stimulation of the pineal gland on a ca 24-h basis (Moore and Klein 1974; Klein and Moore 1979; Reppert et al. 1981). The autonomous nature of the SCN is obvious in constant darkness. Under these conditions the SCN clock continues to drive indole rhythms in the pineal gland; however, these rhythms have a period that is longer or shorter than 24 h. This condition is referred to as 'free running' and is characteristic of all circadian rhythms that are not entrained to the environmental lighting cycle. The molecular basis of this clock is described in detail in this issue by Okamura et al. (2002).

Light influences the SCN clock directly via photoreceptors in the retina and the retinal hypothalamic tract (RHT; see Gillette and Mitchell 2002; Hannibal 2002). The photoreceptors mediating effects of light on the circadian system appear to include classic rods and cones (Bronstein et al. 1987; Nelson and Takahashi 1991; Aggelopolous and Meissl 2000) and a newly described circadian photoreceptor system located in the ganglia cell layer of the retina. Evidence in support of the latter system includes the observation that light alters the circadian system, including pineal melatonin, in mice lacking rods and cones (Freedman et al. 1999; Lucas et al. 1999; for review, see Bellingham and Foster 2002). These photoreceptors appear to comprise a subset of melanopsin-positive retinal ganglion cells that project directly to the SCN and also to other hypothalamic sites including the subparaventricular zone via the RHT (Moore et al. 1995; Kramer et al. 2001; Berson et al. 2002; Hannibal et al. 2002; Hattar et al. 2002; Provencio et al. 2002). At this time in the development of our understanding of the mechanisms involved in the effects of light on circadian function, it appears that the population of photoreceptors mediating this is not fixed and may shift depending on the state of the retina; in addition, it is reasonable to entertain the hypothesis that information from rods and cones might be relayed to the SCN via the melanopsin-positive ganglion cells.

Light has several clear effects on clock function. These include phase shifting and entrainment, which synchronize the clock to the environmental lighting cycle, thereby preventing 'free running'. Another important effect of light is to adjust the duration of the period that the SCN sends stimulatory signals to the pineal gland influencing AANAT activity and melatonin synthesis (Illnerová 1991; Wehr 1991; Arendt 1995). For example, the SCN of animals exposed to 10 days of long nights can stimulate the pineal gland for a longer period than the SCN of animals exposed to 10 days of short nights, as judged by measuring AANAT activity in animals released from light/dark cycles into constant darkness.

The rapid suppressive effects of light described above (Fig. 1) are clock-independent. The suppressive effects of light, also referred to as 'masking', appear to be due to interruption of transmission of signals from the SCN to the pineal gland, i.e., a gating effect downstream of the clock. The precise location of the site at which 'gating' occurs is not clear; gating might occur in the SCN, or other sites innervated by the RHT. Interruption of SCN stimulation of the pineal gland leads to a rapid decrease in AANAT activity and protein (Klein and Weller 1972; Klein 1985; Gastel et al. 1998).

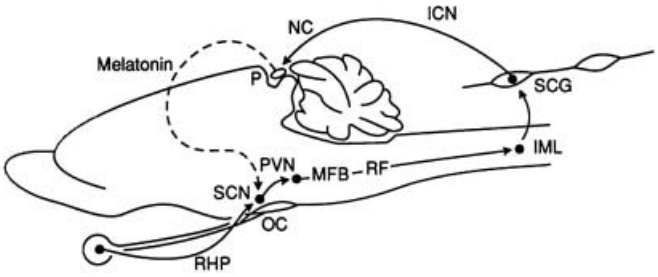

Fig. 2 Mammalian melatonin rhythm generating system. The oscillator regulating pineal function is located in the suprachiasmatic nuclei (*SCN*). Light acts via the retina and a retinohypothalamic projection (*RHP*) to entrain the clock and to adjust the duration of the period that the pineal gland is stimulated. In addition, light acts downstream of the clock system to gate transmission of signals from the SCN to the pineal gland. Monosynaptic connections exist between the SCN and paraventricular nucleus (*PVN*) and between the PVN and preganglionic cells in the intermediolateral cell column (*IML*) that innervate a subpopulation of cells in the superior cervical ganglia (*SCG*). The sympathetic cells course to the pineal gland (*P*) through the inferior carotid nerve (*ICN*) and nervi conarii (*NC*). Stimulation of the pineal gland at night via this system results in the release of norepinephrine, which causes AANAT activity to increase, leading to an increase in melatonin production and secretion. One of the sites of action of melatonin is the SCN. *OC* Optic chiasm, *MFB* medial forebrain bundle, *RF* reticular formation. Modified after Klein et al. (1983a)

Transforming growth factor-α/epidermal growth factor signaling appears to play a role in the SCN and retinal regulation of locomotor activity and sleep (Kramer et al. 2001). This signaling system might also be involved in controlling the melatonin rhythm generating system, both in resetting the clock and in the rapid gating effect of light.

The SCN pineal neural circuit passes through central and peripheral structures (Fig. 2; Klein et al. 1971, 1983a, b; Moore and Klein 1974; Yanovski et al. 1987; Larsen et al. 1998; Teclemariam-Mesbah et al. 1999). Projections from SCN cells innervate cells in the paraventricular nucleus (PVN; for review, see Kalsbeek and Buijs 2002), which in turn send projections through the medial forebrain bundle and reticular formation to preganglionic cells in the intermediolateral cell column of the spinal cord. These innervate a small population of sympathetic cells in the superior cervical ganglia (Bowers et al. 1984), which project to the pineal gland via the inferior carotid nerve and the nervi conarii. Sympathetic innervation of the pineal gland takes the form of an extensive network of norepinephrine (NE)-containing fibers that course through the perivascular space (for review, see Møller and Baeres 2002). Circadian stimulation of these fibers by the SCN→pineal circuit releases NE into this space, where it diffuses to the surface of the pinealocyte (Fig. 3; Brownstein and Axelrod 1974; Drijfhout et al. 1996a, b).

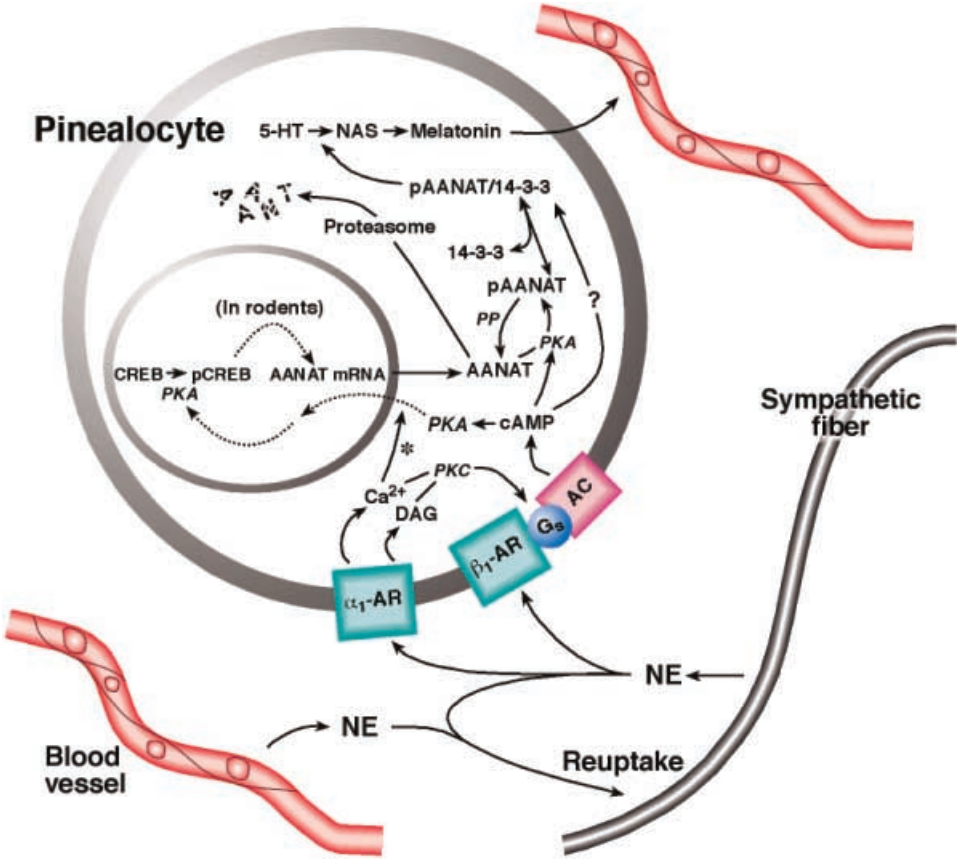

Fig. 3 Adrenergic regulation of AANAT activity and melatonin production. Activation of the neural system outlined in Fig. 2 results in the release of norepinephrine (*NE*) from sympathetic processes coursing through the pineal gland. NE is released into the perivascular space and diffuses to the surface of the pinealocyte where it binds to and activates α_1- and β_1-adrenergic receptors (*AR*). β_1-AR activation results in a G-protein (*Gs*)-mediated stimulation of adenylate cyclase (*AC*); α_1-AR activation elevates Ca^{++} and diacylglycerol (*DAG*), which leads to activation of protein kinase C (*PKC*; Sugden and Klein 1988). The increase in PKC potentiates β_1-AR stimulation of AC (Sugden et al. 1985) through a postreceptor mechanism (Ho et al. 1988a, b; Sugden and Klein 1988), causing a rapid increase in the production of intracellular cyclic AMP. Cyclic AMP acts through multiple mechanisms to increase AANAT activity and melatonin production. In rodents, the abundance of AANAT mRNA is regulated by cyclic AMP; elevation of cyclic AMP causes *AANAT* mRNA to increase more than 150-fold at night. This is driven by phosphorylation of the transcription factor cyclic AMP response element-binding protein (*CREB*) by protein kinase A (*PKA*; Roseboom and Klein 1995). However, in many mammals, including ungulates and the rhesus monkey, *AANAT* mRNA is maintained at high levels throughout the day (Coon et al. 1995; Klein et al. 1997; Schomerus et al. 2000). Translation of *AANAT* mRNA results in production of AANAT protein, which has two fates: destruction by proteasomal proteolysis or protection/activation. In all vertebrates, cyclic AMP-dependent phosphorylation prevents destruction by causing AANAT to bind to 14-3-3 proteins, forming a reversible complex in which AANAT is activated and protected from proteolysis (Ganguly et al. 2001; Obsil et al. 2001). *Question mark* indicates that cyclic AMP appears to activate phospho-AANAT complexed to 14-3-3 through unknown mechanisms (Coon et al. 2001). *Asterisk* indicates that Ca^{++} potentiates cyclic AMP-induced increases in AANAT activity (Yu et al. 1993); the mechanism is not known. The increase in AANAT activity accelerates the conversion of serotonin (*5-HT*) to *N*-acetylserotonin (*NAS*), which is rapidly converted to melatonin by HIOMT (Fig. 1). Melatonin is highly lipophilic and does not appear to be stored. Accordingly, the newly synthesized melatonin is immediately released, raising circulating melatonin levels (Illnerová et al. 1978). The amount of melatonin in the circulation is controlled by production in the pineal gland and rapid destruction by the liver. When neural stimulation of the gland ceases, there is rapid reversal of the activated state, i.e., cyclic AMP decreases, and phospho-AANAT dissociates from 14-3-3, is dephosphorylated, and then is destroyed by proteasomal proteolysis. This leads to a drop in melatonin production and an increase in 5-HT

It is not entirely clear whether the SCN generates positive signals, which are relayed to the pineal gland through the SCN→pineal circuit. Although this is supported by observations that AANAT activity is suppressed in animals with SCN lesions or SCN islands (Moore and Klein 1974; Klein and Moore1979; Reppert et al. 1981), it has also been proposed that the SCN generates signals during the day, but not night, which block PVN stimulation of the pineal gland (Kalsbeek et al. 1996, 1999; for review, see Kalsbeek and Buijs 2002). Although there is disagreement regarding the functional relationship of elements of the SCN pineal circuit, there is no doubt that this circuit mediates the circadian release of NE in the pineal gland; the influence of other neural systems on the daily rhythm in melatonin synthesis in mammals is not clear.

Although there is little or no release of NE from the pineal sympathetic processes during the day, sympathetic processes play a very important day-time role, because they take up NE and epinephrine that diffuse out of the blood into the perivascular space (Fig. 3). This prevents accumulation of catecholamines at levels sufficient to activate the pineal gland (Parfitt and Klein 1976). The importance of this mechanism is evident from studies in which circulating NE is elevated by stress. Under these circumstances, extrapineal-derived NE will activate the denervated pineal gland but not the innervated gland; similarly, treatments with drugs that block reuptake will elevate AANAT activity (Parfitt and Klein 1976; Golden et al. 1988; Oxenkrug et al. 1990).

Neural regulation of pineal second messengers

Norepinephrine controls AANAT activity through actions on adrenergic receptors, which increase the intracellular concentrations of cyclic AMP and Ca^{++} (Fig. 3). Studies in the rat established that elevation of cyclic AMP reflects activation of both α_1- and β_1-adrenergic receptors, which control cyclic AMP production through a combinatorial mechanism that has also been described as 'cross-talk' or an 'AND' mechanism (Sugden et al. 1985; Vanecek et al. 1985, 1986). Norepinephrine activation of β_1-adrenergic receptors increases adenylate cyclase (AC) activity; the concurrent stimulation of α_1-adrenergic receptors by NE potentiates this response through a Ca^{++}, phospholipase C protein kinase C (PKC) mechanism (Ho et al. 1988a, b). The increase in Ca^{++} is due to release from intracellular stores and increased influx (Sugden et al. 1986, 1987; Schomerus et al. 1995; Korf et al. 1997). α_1-Adrenergic receptors also mediate the activation of phospholipase C, which leads to an increase in accumulation of diacylglycerol (Berg and Klein 1972; Ho et al. 1988a, b). The increases in Ca^{++} and diacylglycerol activate PKC, which potentiates β_1-adrenergic activation of AC through actions at a postreceptor site (Sugden et al. 1985; Sugden and Klein 1988).

Second messenger regulation of AANAT

Cyclic AMP plays a highly conserved role in regulating vertebrate AANAT. The importance of cyclic AMP in the regulation of AANAT has been revealed in experiments with several mammalian systems, as well as with pineal glands from fish and chicken. Cyclic AMP and AANAT are linked by two protein kinase A (PKA)-mediated phosphorylation sites (PKA sites) found in all AANAT proteins. These mediate cyclic AMP control of AANAT protein degradation (Fig. 4; Coon et al. 1995; Klein et al. 1997). Cyclic AMP also acts on transcription in some mammals; specifically, cyclic AMP response elements (CRE) in the rodent AANAT gene mediate cyclic AMP regulation of AANAT transcription via PKA-mediated phosphorylation of CRE binding-protein (CREB) (for review, see Stehle et al. 2002).

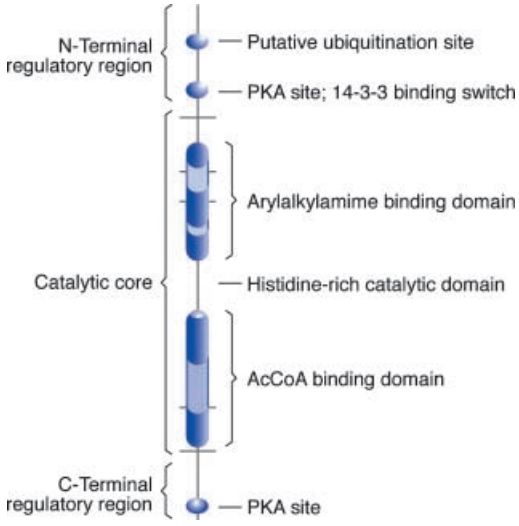

Fig. 4 Structure of arylalkylamine *N*-acetyltransferase (AANAT). Vertebrate AANATs (MW ca 23 kDa) are organized into a central catalytic core, which alone has enzymatic activity, and flanking regulatory regions, each of which has a PKA site. Phosphorylation of the *N*-terminal PKA site converts the 6-residue sequence containing the PKA site (*italicized*) into a 14-3-3 binding motif: *RRHT*LPAN → *RRHpT*LP. The phosphorylated sequence mediates binding of AANAT to 14-3-3 as indicated by functional studies and X-ray crystallographic analysis of the complex (Ganguly et al. 2001; Obsil et al. 2001). The arylalkylamine-binding domain characterizes the AANAT family. The AcCoA binding domain is similar to that found in the acetyltransferase superfamily identified as the 'Motif A/B' superfamily, reflecting two highly conserved motifs in the AcCoA binding domain, or, as the 'GNAT' superfamily, reflecting membership of an important enzyme, GCN5. The structure and function of this superfamily are reviewed in detail in Dyda et al. (2000)

It should also be noted that Ca^{++} has a second, less well understood, combinatorial influence on pineal signal transduction (Fig. 3), mediating α_1-adrenergic potentiation of β_1-adrenergic stimulation of AANAT (Klein et al. 1983b). Ca^{++} potentiates the effects of cyclic AMP (Yu et al. 1993), downstream of cyclic AMP generation. It is not clear whether this effect of Ca^{++} reflects an action on transcription or translation, or on both.

AANAT mRNA: regulation in some but not all mammals

AANAT mRNA levels are elevated continually, day and night, in ungulate and primate pineal glands. Accordingly, changes in transcription play no role in the regulation of AANAT activity in these species (Rollag and Niswender 1976; Namboodiri et al. 1985; Klein et al. 1997; Schomerus et al. 2000). In rodents, however, *AANAT* mRNA increases more than 100-fold at night (Roseboom et al. 1996; Klein et al. 1997). The functional importance of this difference may be linked to the patterns of melatonin production generated in these species. In ungulates and primates, melatonin increases shortly

after lights-off at night. The immediate increase in melatonin may reflect in part the presence of *AANAT* mRNA, which makes it possible for AANAT protein to increase immediately. As discussed below, this increase is due to inhibition of degradation of AANAT protein. The requirement for new synthesis of *AANAT* mRNA in rodents confers a lag period on the increase in AANAT activity. This may be of special importance in tailoring the pattern of melatonin production because it prevents the increase in AANAT activity and melatonin synthesis early in the night period, which is known to be a critical period for effects of melatonin in hamsters (Tamarkin et al. 1980).

The increase in *AANAT* mRNA in rodents is due to cyclic AMP-dependent PKA-phosphorylation of CREB, which binds to the AANAT promoter via multiple CRE elements. The CREB phosphorylation (pCREB) enhances transcription (Roseboom and Klein 1995; Baler et al. 1997).

The degree of enhancement is inversely linked to the abundance of an inhibitory transcription factor, inducible cyclic AMP early repressor (ICER; Stehle et al. 1993; Maronde et al. 1999), which competes with CREB for CRE sites on the *AANAT* gene (Stehle et al. 1993). *ICER* transcription is increased ca 100-fold at night through an adrenergic-cyclic AMP mechanism; however, in contrast, ICER protein exhibits relatively small (ca 4-fold) day/night differences (Foulkes et al. 1996a; Maronde et al. 1999; von Gall et al 2000). The physiological functional importance of daily changes in ICER protein as regards *AANAT* expression is unclear, in part because ICER is not necessary for AANAT to cycle; this was demonstrated using mice in which ICER is not expressed (Foulkes et al. 1996a).

Although a role for ICER in controlling the daily dynamics in *AANAT* mRNA is still a matter of debate, other evidence suggests a long-term role. For example, the amplitude of the increase in *AANAT* mRNA is enhanced in mice not expressing *ICER*, and ICER protein levels change gradually over a period of many weeks (Foulkes et al. 1996a, b). This suggests that ICER may provide a memory of prior stimulation of the pineal gland, and, following prolonged periods of adrenergic stimulation, the peak in *AANAT* mRNA levels gradually decreases because the number of ICER-occupied CRE sites in the *AANAT* promoter has increased, thereby reducing pCREB-dependent transcription (for review, see Stehle et al. 2002).

The decrease in *AANAT* mRNA at the end of the night period has not been studied extensively, but it seems reasonable to suspect that the decrease in cyclic AMP levels at the end of the night period leads to dephosphorylation of pCREB, which turns off *AANAT* transcription. The residual *AANAT* mRNA disappears because transcription decreases and mRNA is destroyed by RNase.

It is of tangential interest to note that other regulatory elements in the *AANAT* gene exist and play a role in regulating expression of the *AANAT* gene (Baler et al. 1999). Specifically, the rat *AANAT* gene contains an E-box sequence that directly interacts with clock genes to control gene expression (Kyriacou and Rosato 2000). This element appears to control the daily rhythm in *AANAT* mRNA in the chicken pineal gland (Chong et al. 2000; for review, see Natesan et al. 2002) and to mediate clock regulation of *AANAT* mRNA in the rat retina (Chen and Baler 2000). Daily rhythms of other clock genes have been reported to occur in the pineal gland, including *mPer1* (in mouse) and *rPer1* (in rat), where they are under adrenergic cyclic AMP control (Fukuhara et al. 2000; Takekida et al. 2000; von Gall et al. 2001). The relationship of these proteins to *AANAT* gene expression has not been established. Finally, AP-1 elements are found in the rat *AANAT* promoter (Baler et al. 1999). These were suspected of mediating effects of Fra-2 protein, which exhibits a ca 100-fold rhythm in the pineal (Baler and Klein 1995); however, this does not seem to be the case, because rhythms in AANAT activity, mRNA, and melatonin synthesis are normal in animals in which pineal Fra-2 is 'knocked down' (Smith et al. 2001).

Regulation of AANAT protein: a highly conserved regulatory mechanism

The mechanism controlling vertebrate AANAT protein (Fig. 4) is the most highly conserved feature of melatonin regulation. It provides a rapid and efficient mechanism through which cyclic AMP controls AANAT activity and melatonin production by regulating the amount of AANAT protein. In brief, cyclic AMP switches the fate of AANAT from destruction to protection and activation (Fig. 5). In the absence of cyclic AMP, AANAT is destroyed by proteasomal proteolysis (Gastel et al. 1998; Zatz et al. 1999; Schomerus et al. 2000; Falcón et al. 2001; Iuvone et al. 2002). Cyclic AMP prevents this through PKA-dependent phosphorylation of the *N*-terminal PKA site, which leads to binding of AANAT to 14-3-3 proteins (Roseboom et al. 1994; Ganguly et al. 2001; Obsil et al. 2001). When cyclic AMP is elevated, it appears that most, if not all, of AANAT is phosphorylated and bound to 14-3-3 in a reversible complex.

Phosphorylation of AANAT promotes binding by converting a sequence with low affinity for 14-3-3 into one with high affinity (Ganguly et al. 2001):

$$\mathrm{RRHT_{31}LP} \rightarrow \mathrm{RRHpT_{31}LP}$$

When bound to 14-3-3, AANAT appears to be protected against proteasomal proteolysis and dephosphorylation by protein phosphatase. In addition, binding increases the affinity of AANAT for low concentrations of 5-HT. The increased affinity appears to reflect the 14-3-3 restriction of the movement of a floppy loop of AANAT in a configuration favoring 5-HT binding (Obsil et al. 2001). X-ray crystallographic analysis indicates that the region containing the *N*-terminal PKA site of AANAT is bound to an amphipathic binding groove in 14-3-3 proteins (Obsil et al. 2001); multiple contacts exist between

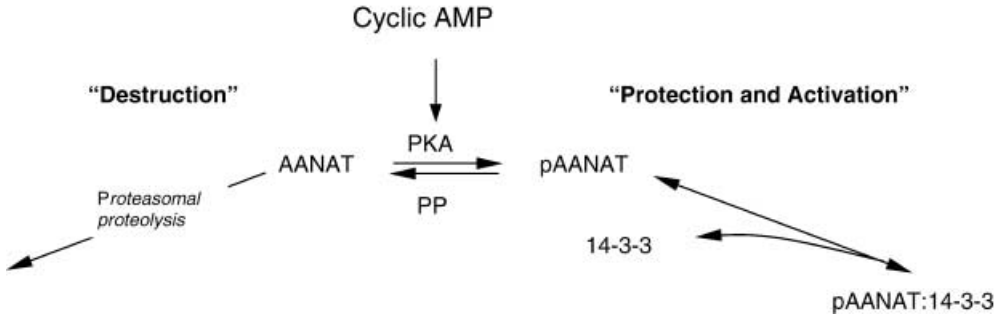

Fig. 5 Scheme showing regulation of AANAT protein by cAMP

pT_{31} and 14-3-3. Although there are numerous contacts involving neighboring residues, the unphosphorylated protein binds poorly to 14-3-3. This indicates that cyclic AMP-dependent phosphorylation of T_{31} functions as a binding switch by adding potential binding contacts.

Although cyclic AMP-PKA mediated phosphorylation favors formation of the complex, the AANAT-14-3-3 complex is reversible (Ganguly et al. 2001). Free phospho-AANAT (pAANAT) can be dephosphorylated, which prevents association with 14-3-3, leading to proteasomal proteolysis. However, in the presence of high levels of cyclic AMP, formation of pAANAT is favored, resulting in formation of the 14-3-3 complex and maintenance of high levels of AANAT activity and melatonin synthesis.

Recent evidence indicates that cyclic AMP can activate pAANAT, apparently while complexed to 14-3-3 proteins in the cell (Coon et al. 2001; Fig. 3). The precise mechanism through which this occurs is not known.

The integrity of the melatonin signal as an indicator of darkness at night

It is of interest to review the components of the melatonin rhythm generating system and to specifically address the question of how each contributes to the integrity of the melatonin signal as an indicator of darkness at night. As described below, multiple layers of mechanisms exist that preclude the inappropriate increase in melatonin production during the day or during periods of the night when melatonin synthesis should not occur.

Suprachiasmatic nuclei

Suprachiasmatic nuclei are the source of circadian neural signals, which drive the rhythm in AANAT activity and melatonin production. The pattern of SCN signals insures the pineal gland can only be stimulated at night. Accordingly, exposure of animals to darkness during the day does not elevate AANAT activity or melatonin production.

It is of interest to note that the relationship between an endogenous circadian clock and AANAT is also seen in the mammalian retina, perhaps in photoreceptor cells (Tosini and Menaker 1996; Chen and Baler 2000; Fukuhara et al. 2001; for review, see Tosini and Fukuhara 2002), and in lower vertebrates, in which the circadian clock is located within the pinealocyte (Bernard et al. 1997; Coon et al. 1998; for review, see Natesan et al. 2002). This underlines the importance of circadian control of melatonin in vertebrate physiology.

Masking effect of light

The suppressive effect of light on neural stimulation of the pineal gland prevents SCN stimulation of the pineal gland at the beginning and end of the night period. This functions to tailor or fine tune the melatonin signal. The rapid effects of light on AANAT activity and melatonin production result in near square-wave melatonin signals, most evident in ungulates and primates, which produce large differences in circulating melatonin within a short period of time. As a result, an animal is able to sense subtle differences in day length.

Neural insulation

The neural pathway from the SCN to the pineal gland appears to be functionally and structurally insulated (Larsen et al. 1998). This appears to prevent significant stimulation of melatonin synthesis via activation of other central neural systems or stress-induced activation of the sympathetic nervous system (Parfitt and Klein 1976). The physiological importance of other neural inputs (Wartman et al. 1969; Møller and Korf 1983a, b; Møller 1999; for review, see Møller and Baeres 2002) in controlling melatonin synthesis has not been well documented.

Reuptake mechanism

Reuptake of NE by sympathetic terminals in the pineal gland has two important effects. One is to rapidly terminate neural stimulation of the gland by sequestering NE in the perivascular space (Parfitt and Klein 1976). As discussed above, the second is to prevent inappropriate stimulation of melatonin production by stress-induced increases in circulating NE.

Dual adrenergic receptor regulation of pineal AANAT at two levels

Dual adrenergic regulation of AANAT occurs at the receptor level, where activation by NE involves α_1-adrenergic Ca^{++}-dependent potentiation of β_1-adrenergic stimulation of AC. This has two advantages. First, it allows for the effects of low levels of NE on AC to be amplified, and it also increases the integrity of the cyclic AMP response to NE by requiring two receptors to be activated. This minimizes the impact that other agonists might have on AANAT and melatonin production. It should be noted that a number of neurotransmitters and related agonists, other than NE, have effects on pineal second messengers (Korf et al. 1998). Although this is of pharmacological interest, the physiological relevance of these observations is unclear because no evidence is available to indicate that any transmitter other than NE is involved in regulating melatonin synthesis at the level of the pinealocyte.

In addition to dual receptor regulation of cyclic AMP, dual receptor regulation of AANAT occurs downstream at a point where effects of cyclic AMP are potentiated by Ca^{++} (Yu et al. 1993). Accordingly, two potentiating mechanisms exist to enhance the effects of NE on AANAT and melatonin production.

AANAT mRNA rhythm in rodents

The absence of *AANAT* mRNA during the day in rodents prevents synthesis of AANAT protein during this period and delays AANAT synthesis at night. This lag period may be important in limiting melatonin production to precise periods of the night, and may be linked to seasonal regulation of the periods during which melatonin receptor regulated systems are sensitive to melatonin (for review, see Pévet et al. 2002; Ross and Morgan 2002; Stehle et al. 2002).

AANAT proteolysis

The rapid destruction of AANAT following cessation of neural stimulation provides a means for very rapidly turning off melatonin synthesis. This improves the melatonin signal by making it nearly square wave; as discussed above, small differences in the duration of the night period can be reliably converted into distinct differences in the duration of melatonin production, making it possible for an animal to accurately detect small differences in day length. This would not be possible if the melatonin production was turned off gradually.

Melatonin destruction

The rapid destruction of melatonin by the liver through 6-hydroxylation and subsequent modification clears melatonin from the circulation. This is essential for the conversion of rapid changes in melatonin production in the pineal gland into rapid changes in circulating melatonin. For example, without this, circulating levels of melatonin would not drop rapidly when melatonin synthesis is reduced by exposure to light.

Summary

In summary, mammals have a complex neural system that converts information about the photic environment, the relative lengths of the day and night, into a chemical signal, melatonin. This system involves the retina, the circadian clock in the SCN, and an SCN→pineal neural circuit that regulates AANAT activity. Neural signals are linked to AANAT activity through multiple intracellular mechanisms. In all mammals, AANAT activity is regulated by cyclic AMP-inhibited proteasomal proteolysis; in rodents, neural control of *AANAT* mRNA expression also occurs, thereby playing an additional role in determining when AANAT protein can be synthesized and melatonin can be produced. Analysis of the components of this system reveals that each contributes to the reliability, integrity, and dependability of the melatonin signal as an indicator of time. This establishes melatonin as the most reliable measure of SCN function in mammals (Lewy et al. 1999; Duffy et al. 2002), to a large degree because other SCN-driven systems, including locomotor activity, temperature, and cortisol are influenced by non-SCN factors. The high integrity of the melatonin signal is consistent with the essential role it plays in seasonal and circadian physiology.

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